Acceleration and Inhibition of Lipid Oxidation by Heme Compounds

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ABSTRACT

The acceleration and inhibition of unsaturated fatty acid oxidation by heme compounds was followed in model systems with an oxygen analyzer. The linoleate to heme molar ratios for maximum catalysis were 100 for hemin and catalase, 250 for metmyoglobin, 400 for cytochrome c and 500 for methemoglobin. With heme concentrations of 2 to 4 times the optimum catalytic amount, no oxidation occurred. Rapid heme destruction was observed with catalyzing ratios of lipid to heme, but with inhibitory ratios a stable red compound formed, believed to be a lipid hydroperoxide derivative of the heme. The ratios of lipid to metmyoglobin for maximum acceleration varied with the pH. Linolenate was much less sensitive to heme catalysis than linoleate. Colorless products of heme degradation had a marked antioxidant effect. A possible mechanism for the antioxidant effect of hemes is advanced, based on the formation of stable heme peroxide complexes or stable heme radicals, or both, during the early stages of oxidation. Prooxidant activity is believed to occur only when the peroxide to heme ratio is so high that the oxidation of the hemes goes beyond the initial stages.

INTRODUCTION

The catalytic effect of iron porphyrins on the oxidative decomposition of polyunsaturated fatty acids was first described by Robinson in 1924 (1). Some of the voluminous work on this important biocatalyst has been reviewed by Tappel (2). The mechanism proposed by Tappel for the prooxidant activity of hemes is based on their known ability to decompose lipid peroxides. New reaction chains are assumed to be initiated by free radicals resulting from the peroxide scission, although direct attack by the heme catalyst on the fatty acid is not ruled out.

That heme compounds can act as antioxidants rather than prooxidants under some conditions was recognized much more recently. Maier and Tappel in 1959 (3), using a Hb catalyst, found that when the concentration of linoleate dropped below a specified level, prolonged induction periods occurred. They attributed this to too little peroxide in the reaction mixture to generate sufficient free radicals for chain initiation. Apparently they did not consider the possibility that Hb itself was acting as an antioxidant.

Banks et al. (4), using increasing amounts of cytochrome c (cyt c) with fixed amounts of fatty acid, found increasing acceleration up to a maximum and then progressive inhibition with still higher concentrations of cyt c. They postulated that the peroxide decomposition brought about by hemes did not result in chain initiating radicals but rather in stable end products and that cyt c became a prooxidant only after being altered in some way by reaction with the lipid peroxides.

Perhaps the most extensive and convincing evidence to date of the antioxidant activity of heme compounds is that of Lewis and Wills (5). These workers demonstrated that Hb, cyt c, hematin and tissue homogenates at high concentrations all had an inhibitory effect on linoleate oxidation, whereas at lower concentrations they catalyzed the oxidation. The concentration of Hb necessary to show antioxidant activity increased with higher concentration of fatty acid. Thus the ratio of Hb to fatty acid determined whether acceleration or inhibition would take place. In the inhibitory range, Hb stopped fatty acid oxidations, catalyzed by cobalt, which were already well under way. No mechanism was suggested.

The obvious implications of these concentration related differences in the effect of heme compounds on lipid oxidation in living tissues and in meats and fish has led us to explore their activity further. By measuring the rate of lipid oxidation with various heme compounds under the same experimental conditions, concentrations for peak acceleration and inhibition could be compared and the relative magnitude of the accelerating effects evaluated. Observations have also been made on changes in heme compounds when brought into contact with linoleate in catalyzing versus inhibiting concentrations and the effect of the decomposed hemes on lipid oxidation.

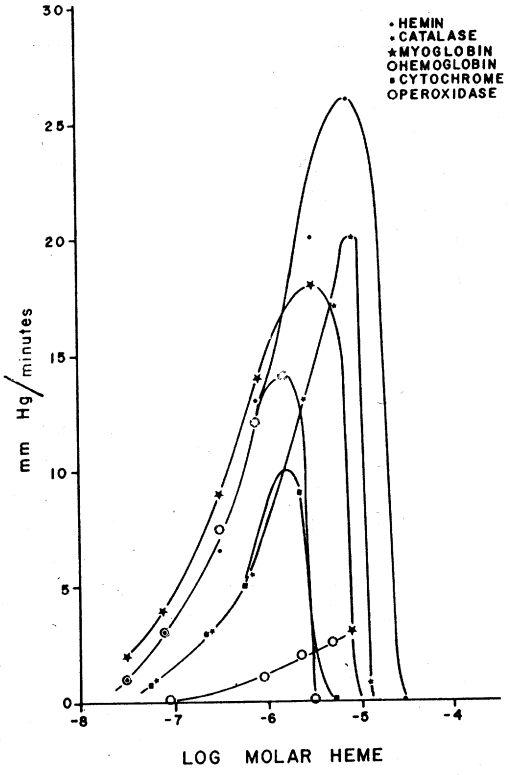

FIG. 1. The effect of varying concentrations of heme compounds on linoleate oxidation, pH 7.4; linoleate concentration, 8.0 x 10^{-4} M.

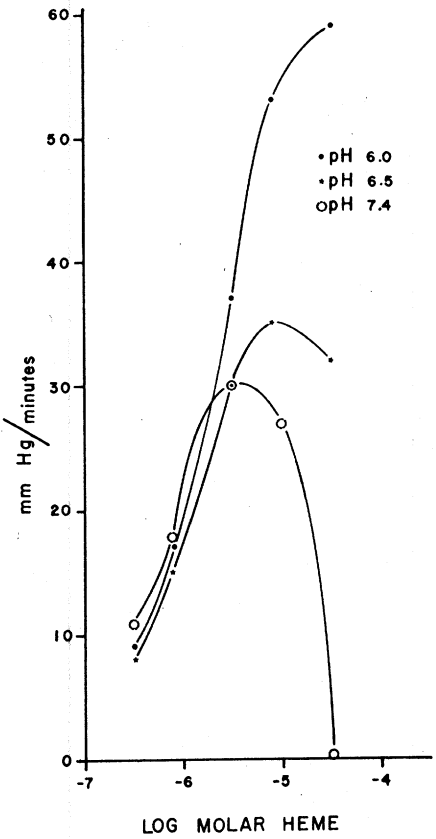

FIG. 2. The effect of pH on the catalytic activity of metMb. Linoleic acid, 8.0 x 10^{-4} M.

MATERIALS AND METHODS

Heme Compounds

Nutritional Biochemicals Corporation supplied hemin (recrystallized), horse metMb (once crystallized and lyophilized), horse radish peroxidase and beef liver catalase (lyophilized). MetHb, bovine (twice crystallized, A Grade) and cyt c, horse heart (salt free, A grade) were obtained from Calbiochem. The methemoglobin (metHb), metmyoglobin (metMb), hemin and cyt c were put into solution at pH 8.5 with M/10 phosphate buffer, the pH adjusted to 7.4 and the solution made to volume with M/10 phosphate buffer, pH 7.4. The peroxidase and catalase were dissolved directly with pH 7.4 buffer.

All of the compounds used were tested by the Hornsey method (6) for acid hematin and all molarities corrected to the equivalent heme basis. The heme contents of the metHb, metMb, cyt c, catalase and peroxidase were 100%, 100%, 68%, 31% and 29% respectively of the theoretical values.

Fatty Acid Emulsions

Linoleic acid (LA) and linolenic acid (LNA) were obtained from the Hormel Institute. Clear aqueous emulsions of the fatty acids were prepared as described by Surrey (7) and adjusted to the concentration and pH desired with M/10 phosphate buffer.

Oxygen consumption in the model systems was measured with a Beckman Model 777 oxygen analyzer. The fatty acid emulsions (43 ml) were transferred to 50 ml Erlenmeyer flasks and stirred magnetically at room temperature. The desired amount of buffered heme compound, contained in 5 ml, was added with a fast delivery pipette. The sensor of the oxygen analyzer was immediately inserted. From the linear slope of the recording, the rate of oxygen consumption by the system was determined and expressed as change in mm Hg PO_2 min. Neither the fatty acids nor the heme compounds oxidized at a measurable rate when tested separately.

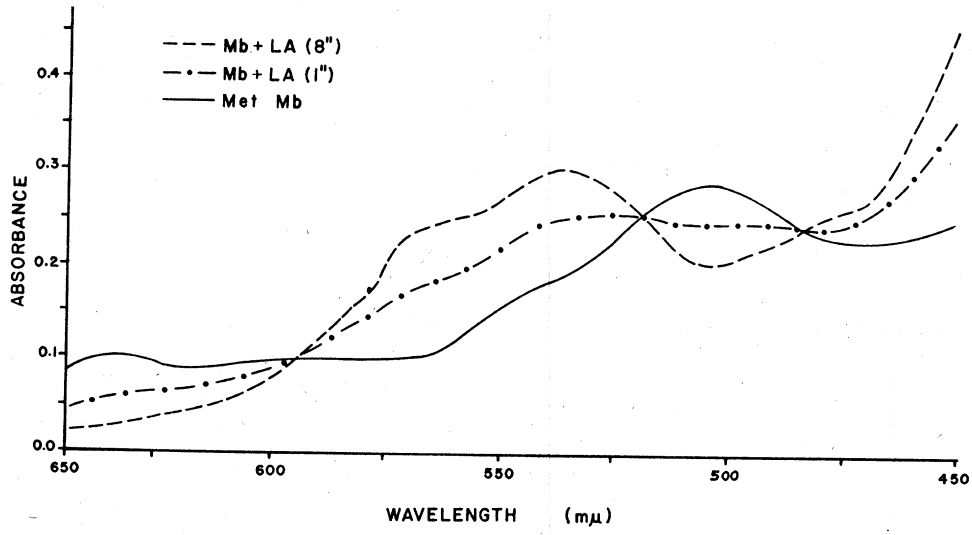

FIG. 3. Changes in the absorption spectrum of metMb when brought into contact with linoleic acid. MetMb, 3.2 x 10^{-5} M.; linoleic acid, 8 x 10^{-4} M; pH 7.4.

RESULTS

Effect of Varying Heme Concentrations

The comparative effect of various heme compounds on the oxidation of linoleate is shown in Figure 1. The peroxidase had only a slight effect at the concentrations tried and was not explored further. Increasing concentrations of all of the other heme compounds progressively increased lipid oxidation up to a maximum value. Further increases in concentration resulted in a sharp drop in lipid oxidation to zero. Uncombined hemin had the greatest range of activity and the highest rate before an inhibitory concentration was reached. All of the protein linked hemes fell within this range.

The data for each heme compound in Figure 1 were obtained on different days, using different linoleate emulsions. When hemin, metMb and metHb were tested at lower concentrations on the same fatty acid emulsion, the initial slopes of all curves were identical, within experimental error. Thus, these three compounds differ in their catalytic action only in that inhibition begins at progressively lower heme concentrations with metHb, metMb and hemin respectively. Catalase and cyt c also resemble each other in initial slopes, but differ in peak concentrations.

The approximate molar ratios of linoleate to heme at the point of maximum catalytic effect (Fig. 1) were 100 for hemin and catalase, 250 for MetMb, 400 for cyt c and 500 for MetHb. At heme concentrations ranging from 2 to 4 times those giving maximum acceleration, oxygen consumption fell off to zero.

Changes With pH

The effect of varying the pH was tried on hemin and metMb. The concentration of hemin giving maximum acceleration was the same at pH 7.4, 6.7 and 6.1 and the general form of the curve was not changed. However, the maximum rate of oxidation increased as the pH decreased. With metMb, not only the rate of oxidation but also the heme concentrations at maximum acceleration increased with decreasing pH (Fig. 2). The crossing over of the curves is a real phenomenon, not attributable to experimental error, i.e., at low metMb concentrations, the highest rate invariably occurs at the highest pH, although the differences are not great.

Linolenic vs. Linoleic Acid

When linolenic acid was substituted for linoleic, again the heme compounds (hemin, metMb and metHb) showed increased catalytic action up to maximal acceleration, followed by inhibition at higher concentrations. The same concentrations of the above heme compounds were maximally effective with linolenic acid as with linoleic, but the concentrations of linolenic acid had to be increased at least five times over that of linoleic to get comparable oxidation rates. The oxidation with 8 x 10^{-4} M linolenic acid was not measurable. A typical set of data with metMb as catalyst is shown in Table I. Obviously the lipid to heme ratio at maximum acceleration will be five times higher for linolenic acid than for linoleic. Tappel (2) pointed out the much lower ratio of heme catalyzed oxidation to autoxidation in linolenic versus linoleic acid.

TABLE I

Effect of MetMb on Linoleic vs. Linolenic Acid

MetMb Concentration (molar)	O_2 Uptake in mm Hg/min Linoleate 8×10^{-4} M	Linolenate 4×10^{-3} M
8.0×10^{-8}	4.0	1.4
3.2×10^{-7}	9.0	3.5
8.0×10^{-7}	14.0	10.5
3.2×10^{-6}	18.0	13.5
8.0×10^{-6}	3.0	5.5
3.2×10^{-5}	0.0	0.0

Observations on Pigment Changes

When catalyzing concentrations of heme compounds were brought into contact with linoleic acid, destruction of the hemes invariably occurred within a few minutes, with complete loss of all peaks in the visible and Soret regions of the spectrum. The concomitant oxidative destruction of the heme catalyst during oxidation of unsaturated fats has been described repeatedly in the literature.

On the other hand, with inhibiting concentrations of heme compounds, new colored reaction products formed upon contact with linoleic acid. These were studied spectrophotometrically with metMb and metHb. With metMb, the brown color changed to red within a few minutes after the addition of the linoleic acid emulsion. Figure 3 shows the changes in the absorption spectrum during the first 8 min. There was only a slight further development of the new peaks at 535 and 563 mμ after an additional 30 min. The new pigment was quite stable; the red color was still apparent when the reaction mixture was held in the refrigerator overnight. Suitable inhibitory ratios of metHb and linoleic acid also produced a red pigment with a spectrum very similar to that of the metMb reaction product. When the red pigments were diluted with linoleic acid emulsion to bring the lipid and heme concentrations into an accelerating ratio, the pigments were quickly destroyed and the lipid oxidized.

The formation of such red pigments in the presence of ferric hemes and lipid hydroperoxides was described by Tappel (8). He interprets the pigments as denatured globin hemichromes. In view of the large concentrations of various nitrogen bases required for hemichrome formation, an alternative interpretation of the pigment as a complex between the ferric hemes and lipid hydroperoxides seems more likely. The formation of such red complexes between H_2O_2 and MetHb (9) and cyt c peroxidase (10) has been described.

TABLE II

Inhibition of Linoleate Oxidation by Heme Degradation Products[a]

Hemes degraded	Inhibition of linoleate oxidation, %
MetHb, 6.7×10^{-6} M	52
MetHb, 6.7×10^{-7} M	39
MetMb, 5.5×10^{-6} M	72
MetMb, 5.5×10^{-7} M	60
Hemin, 5.5×10^{-6} M	73
Hemin, 5.5×10^{-7} M	52

[a]Linoleic acid, 7.2×10^{-4} M, catalyzed by 2.9×10^{-6} M hemin at pH 7.4.

Antioxidant Effect of Heme Oxidation Products

The effect of heme breakdown products on lipid oxidation was tested as follows: hydrogen peroxide was added slowly to solutions of hemin, metHb and metMb until the Soret band could no longer be observed. Approximately 2 ml of 30% H_2O_2 were required to decompose 50 ml of 10^{-5} M heme. The solution resulting from hemin oxidation was clear and colorless; those from metHb and metMb were slightly cloudy after treatment, denoting some denaturation.

Neither the oxidized solutions nor H_2O_2 accelerated oxidation of fresh linoleic acid preparations. When added to rapidly oxidizing linoleic acid, catalyzed by hemin, the decomposed heme products inhibited the oxidation. At concentrations sufficiently high to bring the heme lipid ratio above the normal accelerating range for fresh hemes, the decomposed products brought about complete inhibition. Dilutions of the solutions containing the oxidized products gave partial protection (Table II). Since the antioxidant effect was as great with the oxidation products of hemin as with those of metHb or metMb, it can be ascribed to fragments of the porphyrin ring rather than to the globins. The antioxidant effect of iron free porphyrins has been pointed out by Matsushita and Iwami (11).

DISCUSSION

The data presented here and in the more recent literature suggest that heme compounds act initially as antioxidants, combining with lipid peroxides, as they are produced, to form relatively stable compounds, thus retarding the initiation of new reaction chains. It is only in the presence of large amounts of peroxide relative to the heme that the pigment is altered and free radical reactions are initiated.

The protein moiety confers further stabilizing effects upon the conjugated hemes. Hemin itself had the least antioxidant activity, i.e., the ratio of lipid to heme necessary to overwhelm the heme and convert it to a prooxidant, was generally higher with protein bound hemes (Fig. 1).

King and Winfield (12) and King et al. (13) have pointed out the complex nature of the oxidation of metMb by H_2O_2. Loss of electrons occurs both from iron and from easily oxidizable aromatic amino acids of the protein in the vicinity of the heme. The initial free radical with quadrivalent iron (Mb IV) is relatively stable and capable of combining with other free radicals in the system. Thus it could stop reaction chains in an oxidizing fatty acid system but presumably lacks sufficient energy to initiate new chains by abstracting H from fresh fats. Probably only at a later stage of oxidation, when the porphyrin ring itself is decomposing, are radical intermediates formed of sufficient energy to initiate new reaction chains in oxidizing lipids.

The abrupt shift from antioxidant to maximally active prooxidant with relatively slight increases in the lipid to heme ratios, noted with all of the heme compounds tested (Fig. 1), is rather surprising. The transitory nature of the catalyzing intermediates of the oxidizing hemes and the fact that the end products as well as the initial products of their oxidation are antioxidant in character, probably contribute to this behavior. When the heme concentration reaches a critical level relative to lipid peroxide formation, energetic radicals, which may result from destruction of a fraction of the heme, are removed by reaction with stable heme radicals such as Mb IV and the degraded porphyrin ring eventually supplements the antioxidant action. At lipid to heme ratios below this critical value, the heme is able to contain the free radicals formed, with the result that lipid oxidation is retarded indefinitely. At high ratios the reaction quickly gets out of hand, with wide spread heme destruction and rapid acceleration of lipid oxidation.

The critical ratios of lipid to heme may be expected to vary with the system. The state of dispersion of the unsaturated fat and the heme in heterogeneous systems would certainly influence such ratios, since only the fat in contact with the heme could influence the ratio. A significant pH effect, similar to that noted for metMb (Fig. 2) might also be expected to occur with other conjugated hemes. The relative insensitivity of linolenic as compared to linoleic acid is worthy of further exploration. A more thorough and detailed study of the intermediates formed with each of the hemes and the peroxides of the common unsaturated fatty acids is obviously needed.

Considering the complex structural relationships in animal tissues, speculations as to the probable effect of cellular hemes on lipid oxidation are not highly profitable. Treatments such as the cooking or freezing of flesh foods might be expected to influence such ratios drastically.

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